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Acquisition of broad-spectrum cephalosporin resistance leading to colistin resistance in *Klebsiella pneumoniae*

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An extended-spectrum ß-lactamase (ESBL)-producing and colistin-resistant *Klebsiella pneumoniae* clinical isolate was recovered from a patient who was treated with cefotaxime. This isolate harbored a $bla_{CTX\text{-}M\text{-}15}$ ESBL gene that was associated with an IS*Ecp1* insertion sequence. Tranposition of that tandem occurred within the chromosomal *mgrB* gene, leading to the inactivation of that latter gene and consequently to acquired resistance to colistin. We showed here a co-selection of colistin resistance as a result of a broad-spectrum cephalosporin selective pressure.

Nosocomial infections caused by multidrug-resistant (MDR) *Klebsiella pneumoniae* represent a growing problem worldwide. Use of polymyxins (colistin, polymyxin B) is therefore being currently reconsidered as a last-line treatment option, but also as a first-line treatment in some specific area where MDR organisms are highly prevalent (Italy, Greece, Brazil, ..). However an increasing number of studies are currently reporting resistance to colistin in *K. pneumoniae* (1).

Colistin is a polycationic antibiotic of the polymyxin family interacting with the lipid A moiety of the Gram-negative bacterial lipopolysaccharide (LPS) and consequently disrupting the integrity of the outer membrane. The main mechanisms leading to polymyxin resistance are modifications of the bacterial outer membrane, such as modifications of lipid A with phosphoethanolamine and 4-amino-4-deoxy-L-arabinose (2). The truncation of the *mgrB* gene encoding a negative regulator of the PhoP/PhoQ signalling system, leads to an upregulation of this system, leading to the upregulation of the *pmrHFIJKLM* operon, the synthesis of 4-amino-4-deoxy-L-arabinose, and ultimately to colistin resistance (3, 4). Inactivation of the *mgrB* gene has been identified as a common source of acquired colistin resistance in *K. pneumoniae* with diverse genetic events leading to alterations of the *mgrB*

gene both in human and animal isolates (3-8). In addition, very recently, a plasmid-mediated resistance mechanism has been identified, corresponding to a phosphoethanolamine transferase identified in human and animal enterobacterial isolates worldwide, and also in food products (9-13).

CTX-M-type ß-lactamases are extended-spectrum ß-lactamases (ESBL) reported mostly in *Enterobacteriaceae* (14). CTX-M-15 that had firstly been identified in 2001 from patients hospitalized in India (15) is the most common CTX-M variant worldwide, possessing a significant activity against ceftazidime (16). The $bla_{\text{CTX-M-15}}$ gene is mostly located on large plasmids varying in type and size, but may also be identified on the chromosome of *K. pneumoniae* (17). Acquisition of both chromosomally- and plasmid-located $bla_{\text{CTX-M-15}}$ genes have been shown to be related to the association with insertion sequence IS*Ecp1* (18). IS*Ecp1* is responsible for the mobilization of the $bla_{\text{CTX-M-15}}$ gene and also for its high-level expression (19, 20). More generally IS*Ecp1*-like elements mobilize and promote the expression of $bla_{\text{CTX-M}}$ genes (21).

We report here the identification of a chromosomal insertion of the IS*Ecp1*-$bla_{\text{CTX-M-15}}$ locus into the *mgrB* gene responsible for acquired colistin resistance in *Klebsiella pneumoniae*.

A patient with a previous history of prostate cancer (permanent urinary catheter) and chronic kidney disease was admitted for ischemic heart disease on February 27, 2015, at the Henri Mondor hospital (Paris, France). The following day he was transferred to the intensive care unit (ICU) since he developed a septic shock secondary to a urinary infection with *Morganella morganii* and a bacteremia with *Streptococcus mitis.* Those infections were treated with cefotaxime and gentamicin, and then ofloxacin was given for 12 days. *K. pneumoniae* isolate HM resistant to broad-spectrum cephalosporins and colistin was recovered on March 25, 2015 from a urine sample. Piperacillin-tazobactam combination was consequently initiated and maintained for 15 days. He was discharged but readmitted in the ICU on May 15, 2015, because of a pyelonephritis complicated by an acute renal failure. *K. pneumoniae* isolate HM was again recovered from urine cultures. A treatment with imipenem and amikacin was therefore initiated and maintained for 14 days, and the patient was discharged.

Susceptibility testing was performed by the disc diffusion method according to the EUCAST guidelines, except for colistin (www.**eucast**.org/) (22). MIC of colistin was determined using broth microdilution according to the CLSI guidelines (23). Results were interpreted according to the EUCAST guidelines (22). Although isolate HM was resistant to

broad-spectrum cephalosporins, kanamycin, and gentamicin, and it remained susceptible to piperacillin-tazobactam, cefoxitin, carbapenems, amikacin, ciprofloxacin, trimethoprim-sulfamethoxazole, fosfomycin, and chloramphenicol. The isolates were resistant to colistin with an MIC of 64 mg/l.

Multilocus sequence typing analysis was performed on *K. pneumoniae* HM as described previously (24) and revealed a novel *phoE* allele leading to a new sequence type (ST), namely ST2003 (2-1-62-3-242-4-110) that is differing by only a single allele from ST405.

Sequence analysis of the genes encoding proteins involved in the modification of the LPS (*pmrA*, *pmrB*, *phoP*, *phoQ*, and *mgrB*) was performed as described previously (4). PCR and sequencing identified an integrated fragment of 2,918-bp in-size truncating the *mgrB* gene (Figure). This truncation occurred between nucleotides +21 and +22 and the inserted fragment consisted in an IS*Ecp1*-$bla_{\text{CTX-M-15}}$ compound transposon (19). Noteworthy, even though insertion of IS*Ecp1* has been demonstrated to generate 5-bp target site duplications upon transposition (25), here the IS*Ecp1*-$bla_{\text{CTX-M-15}}$ compound transposon was unusually bracketed by a 7-bp direct repeat sequence (AACCCAC) (Figure).

Conjugation and transformation experiments were performed in order to determine whether another copy of *bla*$_{CTX-M-15}$ gene might also be plasmid-borne in that isolate. Conjugation experiments using *K. pneumoniae* isolate HM as donor and azide-resistant *E. coli* J53 as recipient were therefore performed as described (26), with a selection with ticarcillin (100 µg/ml) and sodium azide (100 µg/ml). No transconjugant was obtained despite repeated attempts. Plasmid extraction from *K. pneumoniae* HM was performed using the Kieser method (27). Electroporation assays were performed into *E. coli* TOP10 using this plasmid extract and selection was done with ticarcillin (100 µg/ml), but no electrotransformant was obtained. In parallel, selection was performed onto colistin (3 µg/ml)-containing plates in order to screen for a putative transferable colistin resistance determinant but no transformant was obtained. In addition, an S1-nuclease pulsed-field gel electrophoresis analysis was performed as described (28) but Southern hybridization with a *bla*$_{CTX-M-15}$-specific probe failed to detect any positive plasmid. Finally, search of the *mcr-1* gene by PCR as described (13) remained negative.

The *bla*$_{CTX-M-15}$ gene was therefore chromosomally-located and inserted into the *mgrB* gene. Noteworthy, the chromosomal insertion of the IS*Ecp1-bla*$_{CTX-M-15}$ tandem into the *mgrB*

gene resulted in acquired resistance to broad-spectrum cephalosporins, and concomitantly to colistin.

Although previous reports identified either premature stop codons, amino acid substitutions or insertions of IS elements as a source of *mgrB* modifications, we identified here an original genetic event truncating the *mgrB* gene that led to acquisition of colistin resistance (3, 5, 6). What we actually observed here was the acquisition of a gene encoding acquired resistance to an antibiotic class leading to acquired resistance to another antibiotic class, which is noteworthy.

Here we report that cefotaxime- and gentamicin-containing regimen were followed by the selection of a *K. pneumoniae* isolate that was resistant to broad-spectrum cephalosporins, gentamicin, and colistin. Noteworthy, the patient never received colistin. We do not know whether this colistin-resistant isolate was selected in-vivo in this patient or whether it was acquired by cross transmission. Considering the increasing rate of CTX-M-producing isolates worldwide, such acquisition of resistance to broad-spectrum cephalosporins being concomitantly a source of acquired resistance to colistin which is now considered as one of the last weapon of our armamentarium, is very worrying.

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Figure legend

Figure. Schematic representation of the *mgrB* gene truncated by IS*Ecp1*/$bla_{\text{CTX-M-15}}$ in *K. pneumoniae* isolate HM. Arrows indicate genes and their respective transcription orientations. IRL, inverted repeat left; IRR, inverted repeat right. The *mgrB* gene truncation is represented by diagonal dashes. *tnpA* corresponds to the transposase encoding gene of IS*Ecp1*. The 7-bp target site duplications are underlined.